Summary

Long-term effects of engineered nanomaterials:

soil ecotoxicological and molecular approaches

The increased application of manufactured nanomaterials (NMs) and their potential release to the environment calls for an assessment of possible chronic effects on environmental targets including the soil ecosystem. Soil organisms play a crucial role in soil ecosystem processes such as decomposition and nutrient recycling, and are exposed to NMs ending up in soil through for example sewage sludge being used as fertilizers or via atmospheric deposition. Consequently, soil invertebrates such as earthworms, isopods, nematodes and springtails may be harmed. It is therefore essential to study the effects of NMs in this group of organisms in order to understand their potential effects in the soil environment. Since stress responses can occur at the physiological as well as the genetic and even epigenetic level, these three levels are examined in different chapters of this thesis. I examined these responses in the springtail *Folsomia candida* (Hexapoda, Collembola) and the potworm *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae), both important model organisms within the field of ecotoxicology due to their ecological relevance, sensitivity to environmental stress, short generation times and ease of culturing in the laboratory. Physiological effects were investigated in long-term multigeneration tests for NM effects on the survival and reproduction of *F. candida.* Genetic effects were assessed by determining gene expression profiles in multiple generations of *F. candida* exposed to two selected NMs. Finally, epigenetic effects were explored by determining DNA methylation patterns in both *F. candida* and *E. crypticus*. Together, these three approaches may help gaining a better understanding of the underlying mechanisms of stress response in soil invertebrates.
I investigated the toxicity of five different engineered nanomaterials (NMs): tungsten carbide-cobalt (WCCo), copper oxide (CuO), iron oxide (Fe_2O_3), organic pigment and multi-walled carbon nanotubes (MWCNTs). These compounds were mixed in with the natural standard LUFA 2.2 soil as a powder in various concentrations. Soluble copper(II), cobalt and iron chlorides were taken as positive controls. Standardized tests according to the guidelines of the Organisation for Economic Co-operation and Development (OECD) were deployed to measure effects on survival and reproduction of the test organisms, and toxicity was related to total metal concentrations in soil and dissolved metal concentrations in pore water. None of the NMs exerted adverse effects on springtail survival and reproduction at concentrations up to 6400 mg per kg dry soil. The Cu, Co and Fe chlorides tested as ionic references caused a 50% decline in springtail reproduction at 981, 469 and 569 mg metal ion per kg dry soil, respectively. The absence of direct (short-term) toxicity of the NMs could partly be explained by the low porewater metal concentrations, suggesting low solubility or slow solubilisation (*Chapter 2*).
In C*hapter 3*, I investigated the phenotypic response of *F. candida* associated with multi-generation exposure to CuO and WCCo NMs, two metal-based NMs that are commonly used in commercial and industrial applications. Survival and reproduction were not affected in any of four consecutive generations of *F. candida* exposed to CuO-NM at concentrations as high as 6400 mg Cu/kg dry LUFA 2.2. soil. On the other hand, WCCo-NM did affect springtail survival and reproduction and from the third generation onwards, with EC50 values between 2400 and 5600 mg NM/kg dry soil. Subsequent recovery was observed in the 5^{th} and 6^{th} generation cultured in clean soil. Histological investigations showed that high concentrations of WCCo-NM (3200 mg/kg) induced tissue damage and disruption of microvilli from the gut epithelial cells. The fact that adverse effects of NMs are only detected after three generations of exposure without observable effects in the previous two generations, suggests that the damage inflicted by WCCo accumulated from one generation to the other, an effect that cannot be recognized in current risk assessment.
Subsequently, I examined the transcriptional responses of *F. candida* associated with multi-generation exposure to CuO and WCCo NMs. Expression of four target genes known to be responsive to stress were investigated by quantitative PCR at different exposure levels (control, 800 and 3200 mg/kg dry soil) over different generations. While the exposures were below toxic threshold concentrations, expression of all genes was significantly affected in response to NM exposure. Moreover, a significant interaction was observed between historical and current exposure and also between historical exposure and generation (*Chapter 4*). We suggest that gene expression assays could detect physiological alterations due to NM toxicity at exposure levels without having phenotypic (reproduction) consequences for any of the springtail generations exposed to CuO-NMs or the first generations exposed to WCCo-NMs.

One way in which physiological effects of stress may be transferred from one generation to the next is through heritable epigenetic markers induced by stress. I therefore explored whether epigenetics-mediated phenotypic plasticity plays a role in the stress response of springtails and enchytraeids exposed to copper. Total cytosine and locus-specific CpG methylation were investigated in *F. candida* and *E. crypticus*, and the possible changes in methylation pattern under toxicant exposure were assessed. LC-MS/MS analyses and bisulfite sequencing were performed to identify the CpG methylation state of the organisms. We showed, for the first time, a total level of 1.4% 5-methyl cytosine methylation in the genome of *E. crypticus*, and an absence of both total cytosine and locus-specific CpG methylation in *F. candida*. In *E. crypticus*, methylation of CpG sites was observed in the coding sequence (CDS) of the housekeeping gene Elongation Factor 1α, while the CDS of the stress inducible Heat Shock Protein 70 gene almost lacked methylation. This confirmed previous observations that DNA methylation differs between housekeeping and stress-inducible genes in invertebrates. DNA methylation patterns in *E. crypticus* were not affected by exposure to copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) mixed in with LUFA 2.2 soil at sublethal effect concentrations that decreased reproduction by 10%, 20% and 50% (*Chapter 5*). Although differences in CpG methylation patterns between specific loci suggest a functional role for DNA methylation in *E. crypticus*, further genome-wide bisulfite sequencing is needed to verify whether environmental stress affects this epigenetic hallmark.

The insights obtained in this thesis have elaborated our understanding of the environmental risks of different engineered NMs. We discovered that none of the selected NMs caused adverse effects on springtail survival and reproduction after single generation exposures up to a high concentration of 6400 mg/kg dry soil, an exposure concentration that is far higher than expected to be found in the environment. Thus, our results seem to take away the concerns about the potential effects of NMs on soil invertebrates. A remaining concern, however, is that long-term NM exposures may have adverse environmental effects as a result of the uptake of NMs by organisms and their fate in the environment. Also, long-term effects of NMs may have impacts at below-organism level, for instance on cell tissues, gene expression and epigenetics, which could result in detrimental effects at the organism and perhaps even the population level. For instance, our results have taught us that adverse effects may only appear after a number of subsequent generations of exposure without observable effects in previous generations, suggesting that damage by NMs accumulates from one generation to the other, an effect which is not normally recognized in current risk assessment. Thus, the possibility that damage can accumulate over several generations requires more attention in the risk assessment, not only of NMs but also of other (persistent) substances.

Furthermore, the observed induction of stress-responsive genes while phenotypic effects are absent suggests that the use of molecular approaches, such as gene expression assays, would be appropriate to test whether organisms are actually affected by exposure to engineered NMs. I recommend to perform multi-generation ecotoxicity analyses for toxicants like NMs that are new and difficult to assess, since long-term behavior of NMs in the environment is practically unknown and the majority of NMs is expected to be undegradable and to persist in the environment for a long time. Considering the complexity of (long-term) NM fate and behaviour and subsequent toxicity effects, I also suggest to perform more dissolution and bioaccumulation studies, rather than reproduction tests, as such studies would provide more information about NM fate and long-term NM behaviour. Finally, as an alternative approach in understanding environmental stress response in soil organisms, I investigated whether epigenetics-mediated phenotypic plasticity (i.e. DNA methylation) plays a role in the stress response of springtails and enchytraeids exposed to copper, a metal that has previously been reported to cause toxic effects in these organisms. We only observed DNA methylation in *E. crypticus* and found that this was not affected by copper exposure, potentially because copper may affect other molecular processes that are very different from for instance cadmium or zinc, and therefore may not affect DNA methylation. It would be an interesting future direction to test other metals to see whether specific metals would cause a specific stress response mechanism in terms of DNA methylation pattern changes. I also recommend to investigate alternative epigenetic mechanisms such as histone modifications. Such mechanisms are not heritable, but have already been observed in other organisms that lack DNA methylation, and may instead be involved in environmental stress response.

Although this thesis builds on our current knowledge regarding nano-ecotoxicity, we are still far away from a complete understanding of multi-generation effects of NMs in the environment.